Supplemental Materials

Smad2 and MRTFB cooperatively regulate vascular smooth muscle differentiation from neural crest cells

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I. Detailed Methods

Cell Culture and reagents

Monc-1 and C3H10T1/2 (10T1/2) cells were cultured as previously described.[1, 2] Smad3 expression plasmids were previously described.[3, 4] Smad2 expression plasmid was a generous gift from Dr. Ying Zhang.[5] MRTFB expression plasmid was provided by Dr. Joseph Miano. Smooth muscle α-actin (α-SMA) and SM22α promoter-luciferase constructs were previously described.[1, 6]

Generation of Smad2 NCC-specific knockout mice

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Georgia. Smad2-floxed (Smad2fl/fl), Wnt1-Cre, and SM22α-Cre mice were previously described.[7, 8] To generate Smad2 deletion in NCCs or SMC, Wnt1-Cre or SM22α-Cre male mice were cross-breed with Smad2fl/fl mice to produce Wnt1-Cre;Smad2fl/+ or SM22α-Cre;Smad2fl/+ mice. The male and female Wnt1-Cre;Smad2fl/+ or SM22α-Cre;Smad2fl/+ mice were then cross-breed to produce Wnt1-Cre;Smad2fl/fl or SM22α-Cre;Smad2fl/fl mice. SM22α-Cre or Wnt1-Cre;Smad2+/+ littermates serve as control. For *in vivo* fate mapping of NCCs, Wnt1-Cre mice heterozygous for Smad2-floxed allele (Wnt1-Cre;Smad2fl/+) were mated with Smad2fl/fl mice carrying a ROSA26 Cre reporter (R26R) allele, which expresses β-galactosidase (β-Gal) upon Cre-mediated recombination.[9] Genotyping and X-gal staining for NCC fate mapping were performed as described.[7, 10-12] X-gal staining was performed as follows: 11.5 days mouse embryos of Wnt1-Cre;Smad2+/+;R26R and Wnt1-Cre;Smad2fl/fl;R26R were stained whole mount for X-gal using LacZ Tissue Staining kit (InvivoGen) according to the manufacturer's instruction. Briefly, the embryos were fixed in fixative solution on ice for 2-3 h and washed in PBS at 4℃ overnight, and then incubated with staining solution at 37℃ for 2-4 hours followed by rinsing with PBS for three times. The development of blue color was observed using a microscope. To observe the inside staining, the embryos were dehydrated and embedded in paraffin. 5 μm sections were cut and counterstained with 0.1% nuclear fast red.

Histomorphometric analysis and immunohistochemistry (IHC) staining

11.5 days of mouse embryos or carotid arteries from adult mice were fixed with 4% paraformaldehyde and paraffin-embedded. Embryos or vessels were cut by serial sectioning (5μm). The sections were stained with hematoxylin and eosin for structural observation or Elastica van Gieson for elastin. For IHC, sections were rehydrated, blocked with 5% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with rabbit anti-Smad2 or α-SMA antibody overnight at 4℃ followed by incubation

with HRP-conjugated secondary antibody. α-SMA staining was visualized by the Vectastain method using NovaRed as a substrate. Smad2 staining was visualized by using Vectastain ABC-AP kit by following the manufacturer's protocol (Vector Laboratories). The vessel sections were counterstained with hematoxylin.

Preparation of shRNA adenoviral vector

Adenoviral short hairpin RNA (shRNA) target sequences were ATG GAG CTG GTG GAG AAG AA for MRTFB and TGG TGT TCA ATC GCA TAC TAT for Smad2. Double-stranded DNAs coding MRTFB shRNAs were cloned into pRNAT-H1.1/Adeno shuttle vector (Genscript). Adenovirus expressing Flag-tagged MRTFB was constructed by cloning human MRTFB cDNA into the Xho I site of pShuttele-IRES-hrGFP-1 (Agilent) and was confirmed by sequencing. Adenovirus was packaged in 293 cells (Agilent) and purified by CsCl2 gradient ultracentrifugation as previously described.[13, 14] Viral particle titer was determined by plaque assay. Adenovirus expressing Smad3 shRNA was previously described.[15] For adenoviral transduction, Monc-1 cells were transduced with 100 moi of adenovirus expressing control, MRTFB, or shRNA for 24 to 48 hours.

Reverse Transcription-PCR (RT-PCR) and quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instruction. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad). RT-PCR was performed using Bio-Rad C1000 thermal cycler. qPCR was performed in MX3000P qPCR machine using SYBR Green qPCR Mastermix (Agilent). The primer sequences were as follows: Smad2: 5'-CCG GCT GAA CTG TCT CCT AC -3' (forward) and 5'-GCA GAA CCT CTC CGA GTT TG -3' (reverse); Smad3: 5'-CTG GGC CTA CTG TCC AAT GT -3' (forward) and 5'-GCA GCA AAT TCC TGG TTG TT-3' (reverse). The primers used for VSMC markers were described previously.[2, 16]

Western blotting

Neural crest Monc-1 cells were lysed in RIPA lysis buffer. Carotid arteries were homogenized using a tissue homogenizer in RIPA buffer containing protease inhibitor mix (Sigma). Samples were separated on SDS-polyacrylamide gels, and electrotransferred onto PVDF membranes (Bio-Rad). The membranes were incubated for 16 h at 4°C with antibodies against α-SMA (Millipore), SM22α (Abcam), Smad2 (Cell Signaling), or α-tubulin (Sigma) in blocking buffer containing 5% milk followed by incubation with HRP-conjugated secondary antibody (Sigma).

Transient transfection and luciferase assay

Monc-1 and 10T1/2 cells were plated at 2×10^5 cells/well in 12-well plates and incubated at 37°C in a 5% CO_2 incubator until 80% confluence. Cells were then transiently transfected (in triplicate) with LipofectAMINE LTX Plus according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA). Luciferase assay was performed as described previously.[1, 17]

Co-immunoprecipitation assay (Co-IP) and immunoblotting analysis

Monc-1 cells were transduced with adenovirus expressing Flag-tagged MRTFB followed by vehicle or TGF-β treatment. Cells were then lysed with ice-cold lysis buffer containing protease inhibitor mix. The lysates were incubated with IgG or Flag antibody for one hour and then protein-A/G agarose at 4°C for 12 hours. The immunoprecipitates were pelleted, washed and subjected to immunoblotting using Flag or Smad2 antibody as described previously.[15, 18]

Immunofluorescent staining and confocal microscopy

Adenovirus expressing Flag-tagged MRTFB was transduced with adenovirus expressing GFP or Smad2 shRNA into Monc-1 cells for two days followed by vehicle or 5 ng/ml of TGF-β treatment for 2 hours. The cells were then fixed and incubated with rabbit anti-Flag antibody (Sigma), followed by incubation

with TRITC-conjugated secondary goat anti-rabbit IgG as described previously.[17, 19] MRTFB nuclear translocation was observed with confocal microscopy. DAPI stains nuclei.

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as described previously (34). Monc-1 cells were transduced with adenovirus expressing GFP, MRTFB, or MRTFB shRNA followed by TGF-β treatment for 2 hours. Chromatin complexes were immunoprecipitated with 3 μg Smad2 antibody or IgG (negative control). Semi-quantitative PCR and qPCR were performed to amplify the SM22α promoter region containing functional Smad binding element (SBE) using the following primer set: 5'- TCT GCC CCA GCC CAG ACA CC -3' (forward) and 5'- CCC ACA GCC CTT CTG CTC CC -3' (reverse).

Statistical analysis

All values are expressed as mean ± SEM. Data were analyzed using ANOVA with pairwise comparisons between groups. P values < 0.05 were considered statistically significant.

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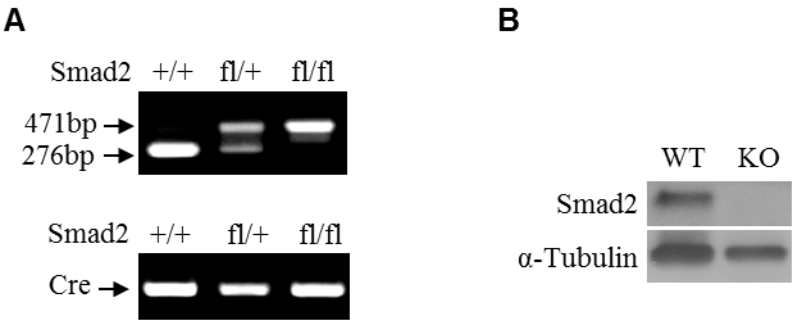
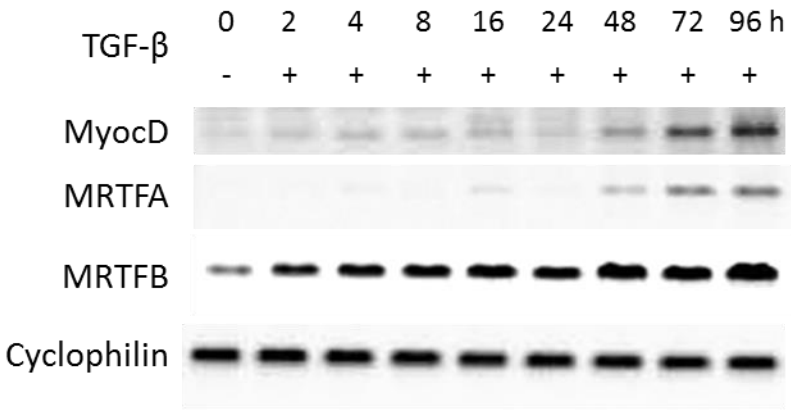
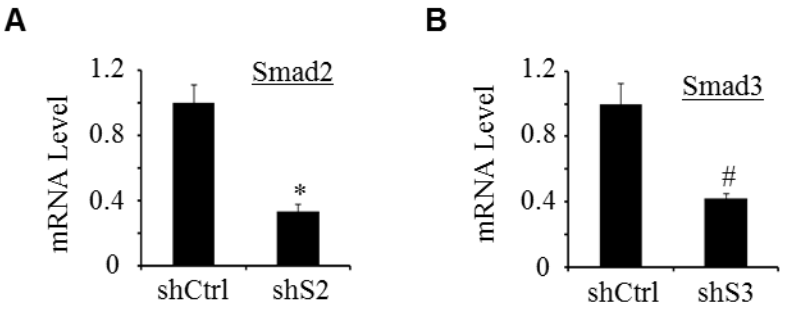
II. Supplemental Data



Online Figure I. (A) Genotyping of Smad2-floxed mice mated with Wnt1-Cre mice was detected by RT-PCR using mouse tail biopsy. 471bp band indicates homozygous Smad2floxed mice. 276bp band indicates wild type mice. Appearance of two bands indicate heterozygous Smad2floxed mice. **(B)** Western blot confirmed that Smad2 protein was expressed in wild type (WT) but not in the Smad2 KO carotid arteries.



Online Figure II. Myocardin (Myocd), MRTFA, and MRTFB protein expression in TGF-β-treated Monc-1 cells. Monc-1 cells were treated with TGF-β (5 ng/ml) for the times indicated. Western blot were performed. Myocd and MRTFA were induced 48 h after TGF-β treatment while MRTFB was induced 2 h after the treatment.



Online Figure III. shRNA knockdown efficiency of Smad2 and Smad3. Smad2 (A) and Smad3 (B) were knocked down by Smad2 (shS2) and Smad3 shRNA (shS3), respectively. The knockdown efficiency was detected by qPCR.